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PULSE AND TRAPEZOIDAL VOLTAGE CLAMP APPLIED TO JURKAT CELLS: A T-LYMPHOCYTE CELL LINE

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TECHNICAL REVIEW AND APPROVAL
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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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The whole cell patch technique was applied to Jurkat cells, a human leukemic T-lymphocyte cell line. From a holding potential of 70 mv, a sequence of depolarizing square voltage pulses ranging from 0 to 130 mv., spaced 10 mv apart and of duration 190ms., was applied to the cell membrane and the resulting currents measured. Peak currents as high as two nanoamps could be obtained from some cells while other cells showed barely detectable currents in response to 130 mv. depolarizing pulses. The shape of the current responses to the large depolarizing pulses varied greatly from cell to cell. Some cells showed current vs. time curves with broad peaks with latency to the peak of about 12 ms and other cells showed narrow peaks with latency to the peak of less than 5 ms. The reasons for this variability are not clear but may reflect the stage of the cell cycle when the patch was made as the cultures were in log phase during our studies.

In addition to square pulses, trapezoidal voltage wave forms were applied to the cell and the evoked currents were measured. In some experiments of this sort there appeared to be a negative resistance region

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of the current voltage curve. Also, it may be possible to interpret the current vs. time records evoked by the trapezoidal voltage wave forms in terms of a variable capacitance. These points may become clearer when adequate electrical models of preparation are made.

Under zero current clamp, the membrane voltage fluctuates with time. This phenomenon may be related to the negative resistance sometimes observed.

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ACKNOWLEDGEMENT

We thank Mr. Alfred Black for giving us Jurkat cells for electrophysiological studies.

INTRODUCTION

Using the patch clamp technique of Neher and Sakmann (1) two laboratories independently discovered a voltage sensitive potassium channel in T lymphocytes (2,3), also called T-cells. The following observations suggest that these channels are important to the cell. Quinine, 4 amino-pyridine (4AP), and tetra ethyl ammonium (TEA) are potassium channel blockers. The concentrations of quinine, 4AP, and TEA required to block this channel are about the same as the concentrations required to inhibit thymidine uptake in PHA stimulated cells. This suggests that these channels participate in mitogenesis. Because of their possible importance they have been extensively studied.

The lymphocyte voltage sensitive potassium channels have been divided into three types, type n, type n', and type l. These types differ in the magnitude and the time course of the current through the cell membrane evoked by a given depolarizing pulse from a holding potential near -80 millivolt, and in their sensitivity to various blocking agents (4). For a review of the electrophysiology of white blood cells see (5).

In this report a qualitative description will be given of the time varying currents evoked by two sorts of time varying voltages, steps and trapezoidal voltages, applied to the lymphocyte cell membrane. (Definitions supplied below.) The results with trapezoidal voltages suggest that the membrane capacitance may not be a constant or that conventional electrical models of cell membranes need revision. Also, fluctuations in

the membrane potential will be shown. .

Jurkat cells (cell line Eb-6), a T-cell immortal cell line, were used in this study. Previous work suggests that the voltage sensitive potassium channels in these cells are type n (6).

METHODS

Jurkat cells were grown in a standard RPMI-1640 medium with 5% fetal calf serum (200 mM), L-Glutamine and Pen-Strep. These cells were isolated from culture via centrifugation at 1100 rpm for six minutes. The cells were then washed with extracellular solution composed of (in mM): 145 NaCl, 4.5 KCl, 1.6 $CaCl_2$, 1.1 $MgCl_2$ and a 10 mM concentration of HEPES buffer, pH=7.3 with NaOH. The cells were then spun down again at 1100 rpm for six minutes and then resuspended in sufficient extracellular solution to give a final concentration of 600,000 cells per ml. in the culture dish. Cell viability was estimated to be greater than 95% by the trypan blue exclusion test.

There are two methods in common use for studying the electrical properties of biological membranes; the voltage clamp and the current clamp. In the voltage clamp the voltage across the membrane is made to follow a predetermined function of time and the resulting current as a function of time is measured. In the current clamp the current is made to follow a predetermined function of time and the resulting voltage as a function of time is measured. The patch clamp technique is a means of doing these two things.

The most common current clamp is to set the current to zero.

This is equivalent to measuring the potential difference across the membrane. In this report the only current clamp used was the zero current clamp.

The basic tool was the patch pipette, a glass capillary drawn by a micropipette puller from fine glass tubing. (We used a Browm Flaming pipette puller and special glass tubing called "Accufil-90 Micropipet", sold by Clay Adams, Division of Becton, Dickinson and Company, Parsippany N.J. 07054.) The pipette tip, about two microns in diameter, was made square by the pipette puller as it pulled the pipette and made smooth by fire polishing. (Fire polishing was not always necessary because, for unknown reasons, successful patches often could be achieved with out fire polishing). The pipette was filled with a special solution whose ionic composition, similar to that of the cytosol, was in mM 140 KCl, .1 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES buffer, pH=7.3 with NaOH and 1.1 EGTA. (The EGTA buffered the calcium ion concentration.) Just before filling the pipette we filtered the solution with an Acrodisc 13 .2 micron filter that fitted on a syringe.

For patching, the cells were placed in a tissue culture dish that was then mounted on the stage of an inverted microscope and viewed with Hoffmann Modulation optics.

The reference electrode was a salt bridge that was filled with a solution of the same composition as the solution bathing the cells. One end of the salt bridge dipped into the bathing solution in the culture dish and the other end made contact with

a silver-silver chloride electrode connected to the ground of the patch clamp amplifier. A source of unwanted potential was caused by asymmetry in the silver-silver chloride electrodes and the solutions with which they made contact. This potential was a few millivolts and could be balanced electronically so that the error in the potential clamp across the membrane was no more than 1 mv.

All experiments were performed at 21°C.

The pipette was inserted into a holder in which a chlorided silver wire was inserted into the big end of the pipette. The other end of the wire was connected by means of a BNC connector to the head stage of the patch clamp electronics. The head stage was mounted on a micromanipulator that was part of a Neher patch clamp tower. The holder was placed in the head stage and the pipette tip lowered by means of the micromanipulator into the solution containing the cells. For successful patching the resistance of the pipette should be between 1 and 4 megohms. The tip was then maneuvered until it barely touched a cell. There was a port on the side of the pipette holder to which was attached with flexible plastic tubing a 2 ml micrometer syringe (Gilmont Instruments Inc., obtained from Thomas Scientific) A slight positive pressure was applied to the inside of the pipette by turning the syringe drive one revolution in the direction of increasing pressure. In preliminary experiments a weak solution of trypan blue was placed in the pipette so one could visualize the outward flow from the pipette. The solution, as it left the

pipette, took on a ropy appearance, much like the smoke from a lighted cigarette resting in an ash tray. This indicated a slight turbulence in the solution as it flowed from the pipette.

During the patching process the resistance of the pipette was continuously monitored by the current evoked by a 500 Hz, 4 mv. square wave applied to the pipette. This current was converted to sound by an audio amplifier and speaker connected to the output of the patch clamp amplifier. While continually visualizing the cell with the inverted microscope the pipette was maneuvered to be sufficiently close to the cell so that one could detect a slight drop in the intensity of sound from the audio monitor due to a slight increase in the pipette resistance. When this happened the syringe drive was turned one revolution in the direction of decreasing pressure. If the resistance increased to above a gigaohm a successful seal was judged to have been made. If not the syringe drive was turned in the direction of decreasing pressure (increasing suction) until a sudden increase in resistance was observed. Usually no more than 4 turns on the syringe drive were required to achieve a seal. When a seal is obtained the pressure inside the pipette is returned to zero by turning a bleeder valve that is inserted between the end of the syringe and the plastic tubing leading to the pipette holder. Upon a successful seal the preparation is said to be in the on cell configuration.

We believe that the gentle outward flow of solution from the pipette as it approached the cell washed away debris from the

cell so that a clean surface was available to the pipette for patching.

The on cell patch has been extensively used to study the opening and closing of single channels, but for this report it served as an intermediary step for the production of a whole cell patch. After the on cell patch was formed an additional sudden suction, obtained by rapidly turning the knob on the micro syringe, caused the cell membrane covering the tip to rupture so that the inside of the pipette came in contact with the inside of the cell. During the rupturing process a train of small pulses was applied to the membrane. Successful rupture was signaled by a sudden increase in the size of the current transient through the pipette caused by the small voltage jump. This transient was the current charging the cell membrane capacitance. Immediately after rupture the pressure inside the pipette was returned to the ambient pressure by opening the bleeder valve. After a few minutes the inside of the pipette equilibrated with the inside of the cell, and measurements could be made. A successful whole cell patch had been achieved.

We used a patch clamp amplifier and associated circuits, called the EPC-7, designed by Prof. Sigworth and supplied by Adams and List Associates.

The EPC-7 has a 3 pole Bessel filter whose band pass is D.C. to 3000 Hz. For all records in this report the filter was on.

We used the pclamp soft ware package and the TL-1 interface, both supplied by Axon Instruments Inc., in conjunction with an

IBM AT personal computer to produce the voltage pulses and to collect the data from EPC7.

RESULTS AND DISCUSSION

Most workers, who study the voltage sensitivity of lymphocyte channels by the whole cell patch technique, use a protocol of voltage pulses similar to that of Hodgkin and Huxley in their famous 1952 series of papers on ionic conductances in the squid giant axon membrane (7). Protocols of this type, which we call HH protocols, are widely used in whole cell patch clamp studies on many different kinds of cells.

Membrane potential is usually specified as potential inside the cell minus potential outside the cell. The resting potential of most cells is between -70 mv. and -30 mv. In our voltage clamp experiments the membrane potential was held at -70 mv. except when a time dependent voltage wave form was presented to the cell. Pulses that make the membrane potential more positive are called depolarizing pulses.

From a holding potential of -70 mv. we made a sequence of 14 depolarizing pulses, each 190 ms in duration, and each 10 mv more positive then the preceding pulse. The time between successive pulses was 5 sec. This time is sufficiently large so that the cell membrane, after a given pulse, has enough time to return to the steady current produced by a holding potential of -70 mv. Figures 1 through 5 show the responses evoked by the above protocol for 5 cells. The bottom panel of each figure shows the pulses. The pulses are superimposed for ease of viewing. The

top panels show the currents evoked by the pulses also superimposed for ease in viewing. Time scales for top and bottom panels are the same.

The record of each response consists of 2048 data points, each separated by 100 microseconds. The record duration is thus 204.8 milliseconds.

The response amplitude increases monotonically with the voltage pulse amplitude. Because of this we did not label the responses with the voltage pulses that produced them. In all the sequences of records the first depolarizing pulse has zero amplitude so the current was not affected by this pulse. By counting the responses one can link the responses with the voltage pulses that produced them. We call the above protocol the HH protocol after Hodgkin and Huxley.

Upward deflection in the current record represents positive current flowing from the interior of the cell, through the cell membrane, to the outside of the cell. This convention is also used for the trapezoidal clamp defined below.

We number the data points 1 to 2048. In the HH protocol the step is turned on at data point 77 and is turned off at data point 1977. A step in the membrane voltage from -70 to -60 mv did not appear to activate the channel. Record 2 for each HH sequence is the response to this step. To estimate the resistance of the preparation in the absence of potassium channel activity we took from record 2 the mean of the values of data points 26 to 76 and the mean of the values of data points 1926 to

1976 and divided the difference of these two means (whose units are picoamps) into 10 millivolts. The result is the resistance of the preparation in gigaohms in the absence of potassium currents. This resistance is often called the leakage resistance.

In the experiments discussed here we did not coat the electrode with sylgard to reduce noise or compensate for stray and membrane capacitances. Because of the large ionic currents that occur in most cells the error from noise in the measured current was negligible. In some records, produced by the HH protocol, the overloading of the input stage, because of the uncompensated membrane capacitance, may have led to some distortion of the current records in the first few hundred microseconds after the step in membrane voltage. The resistances in gigaohms of the preparations shown in Figs. 1, 2, 3, 4, and 5 were respectively 6.52, 4.05, 1.37, 1.53, 0.57. The most striking feature is the extreme variability of the response. The response in Fig. 1 is mostly due to charging and discharging the membrane capacitance while the response in Fig. 4 shows an ionic conductance with a large peak amplitude of nearly 2 nanoamps. Some of this variability may result from size difference in cells. Let I(t) be the current evoked by the voltage clamp as a function of time. Some preparations, at large depolarizing steps, show "pointy peaks" i.e. d^2I/dt^2 is large at the maximum of I(t) (Figs. 2, 3). Other preparations show "rounded peaks", i.e. the d^2I/dt^2 is small at the maximum of I(t) (Fig. 4). Still

other preparations show the peak of I(t) for large depolarizing pulses to be coincident with the capacitative transient (Fig. 5). These differences in I(t) might be caused by variations from cell to cell in the channel types and the number of channels of each type present in the cell membrane. This is a reasonable supposition in view of the work of Lewis and Cahalan mentioned above. The cells may be all the same and these differences in the voltage clamp records reflect variation in the patching of the cells by the experimenter. (The seal resistance might vary from preparation to preparation. Considering the wide variation in the preparation resistance this idea is plausible.) The cells used in the experiments described above are in log phase. The types of ionic currents present in a given cell may reflect the stage of the cell cycle of this cell. Variations in the ionic currents from cell to cell may be caused by these and other effects.

We have also used another form of voltage clamp which we call the trapezoidal clamp (bottom panels of Figs. 6, 7, 8). It consists of an upward ramp, followed by a constant depolarizing voltage of 30 mv. lasting 20 ms., followed in turn by a downward ramp. The slope of the downward ramp is equal and opposite to the slope of the upward ramp. The pclamp software approximates these ramps by a flight of stairs of 300 steps, each step being 1/3 of a millivolt high and 20 microseconds long. There are 2048 data points for each record in which the trapezoidal response occurs and the A/D rate is 20 micro seconds per point so the duration of

each record is 40.96 milliseconds. Other workers appear to have not used the trapezoidal clamp to study lymphocyte membranes.

Figs. 6, 7, and 8 shows the responses in 3 cells to the above trapezoidal clamp. The bottom panel of each figure shows the trapezoidal clamp; the top panels shows the currents evoked by the trapezoidal stimulus. Time scales for top and bottom panels are the same.

The most noticeable feature of these records is that the points where the magnitude of dI/dt is greatest is where dV/dt abruptly changes. This is doubtless due to abrupt changes in dV_c/dt where V_c is the voltage across the membrane capacitance C. Since the displacement current through the capacitance is $C(dV_c/dt)$ the abrupt changes in dV_c/dt causes large changes in the displacement current. This causes large changes in I, the total current going through the preparation. At present, the fraction of I that is the displacement current is unknown.

The record of Fig. 6 was taken 1 minute after the record of Fig. 1, both records being from the same cell. The jumps in the record of Fig. 6 occurring at A, B, C, and D are all about the same size and probably are caused by the turning on and off of the displacement current that accompanies the turning on and off of the voltage ramp. Figs. 1 and 6 suggest that there are no or few active channels in this cell.

Fig. 8 shows a record, produced by a trapezoidal clamp, from a cell where a fair number of channels are present. Points A, B, C, and D are where discontinuities in dV/dt occur. Notice that

the jumps in the current record at B are smaller than the jumps at A, C, and D. Notice that dI/dt is smaller before the arrow than between the arrow and B. This may mean that channels are closed in the first time interval and open during the second time interval when the depolarization of the membrane is sufficiently large to activate the channels. The smaller jump at B may have a simple explanation when the correct electrical model is found or it may have a more complex explanation involving a coupling of a decrease in cell membrane capacitance with potassium channel activation. We realize the second suggestion is hard to believe.

Fig. 7 shows the record from an experiment whose protocol is the same as that of Fig. 8 Note the presence of a negative resistance indicated by the arrow in Fig. 7 Such records, although not common, do occur and suggest that the cell membrane might show oscillations if the conditions are right. This idea is supported by Fig. 9 which shows a section from a long continuous strip of a voltage record taken under zero current clamp. (Upward deflection represents depolarization). Low frequency oscillations are clearly visible. Such oscillations have been described by Maltsev (8). Negative resistance and oscillations have been found in macrophages whose membrane potential was monitored with ultra micro pipettes (9,10,11).

Earlier work suggests that the HH protocol activates voltage sensitive potassium channels in T cells (4). In our work that is most likely true. The negative resistance that we occasionally observe may represent another ionic conductance although at this

time this can only be a speculation.

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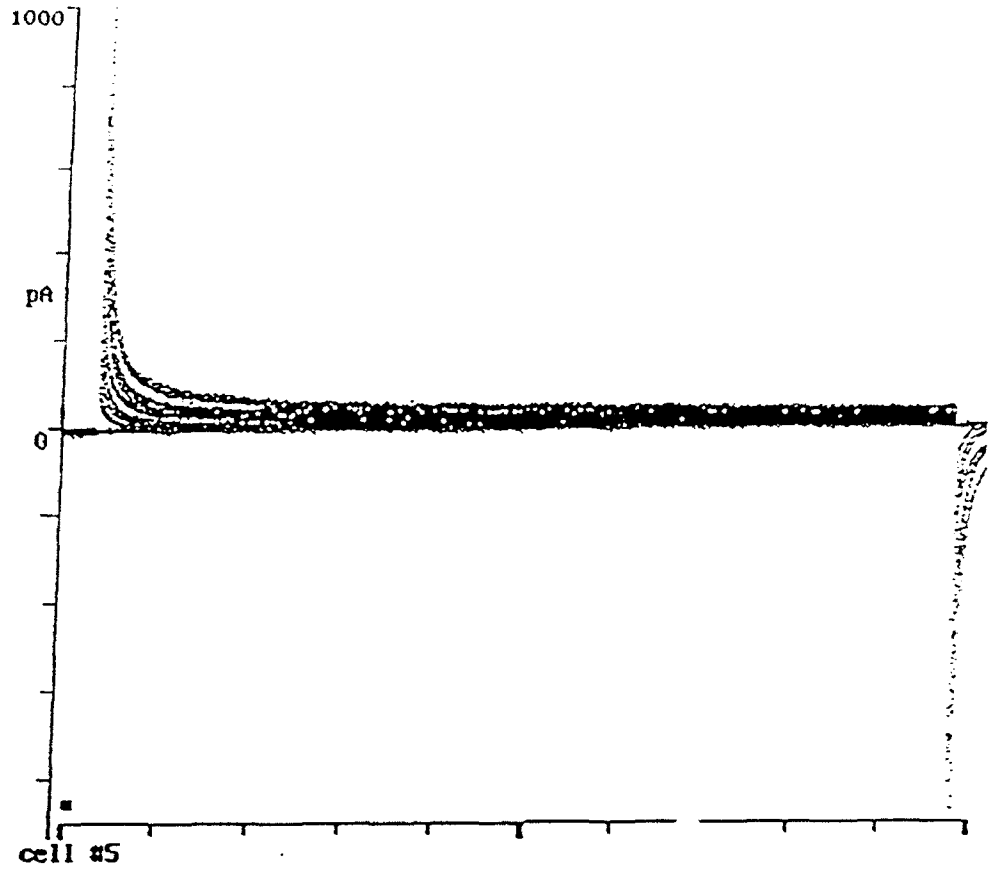
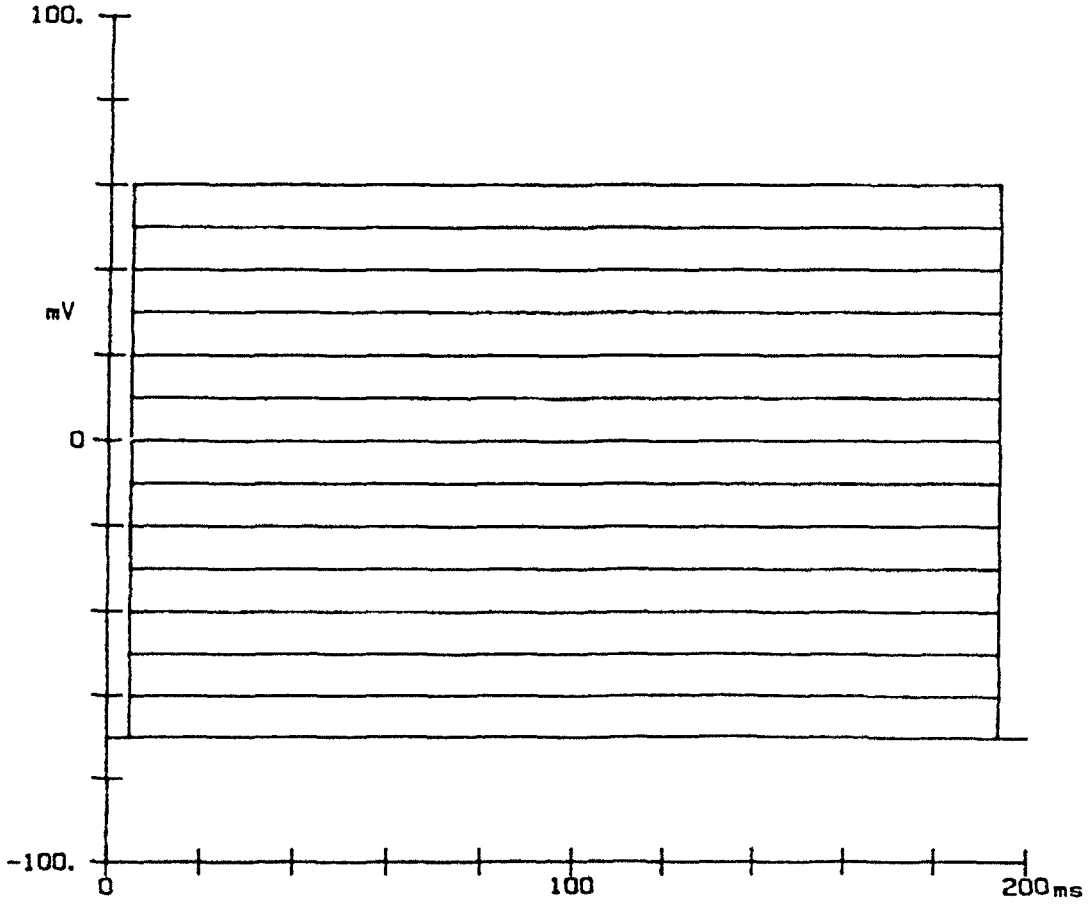
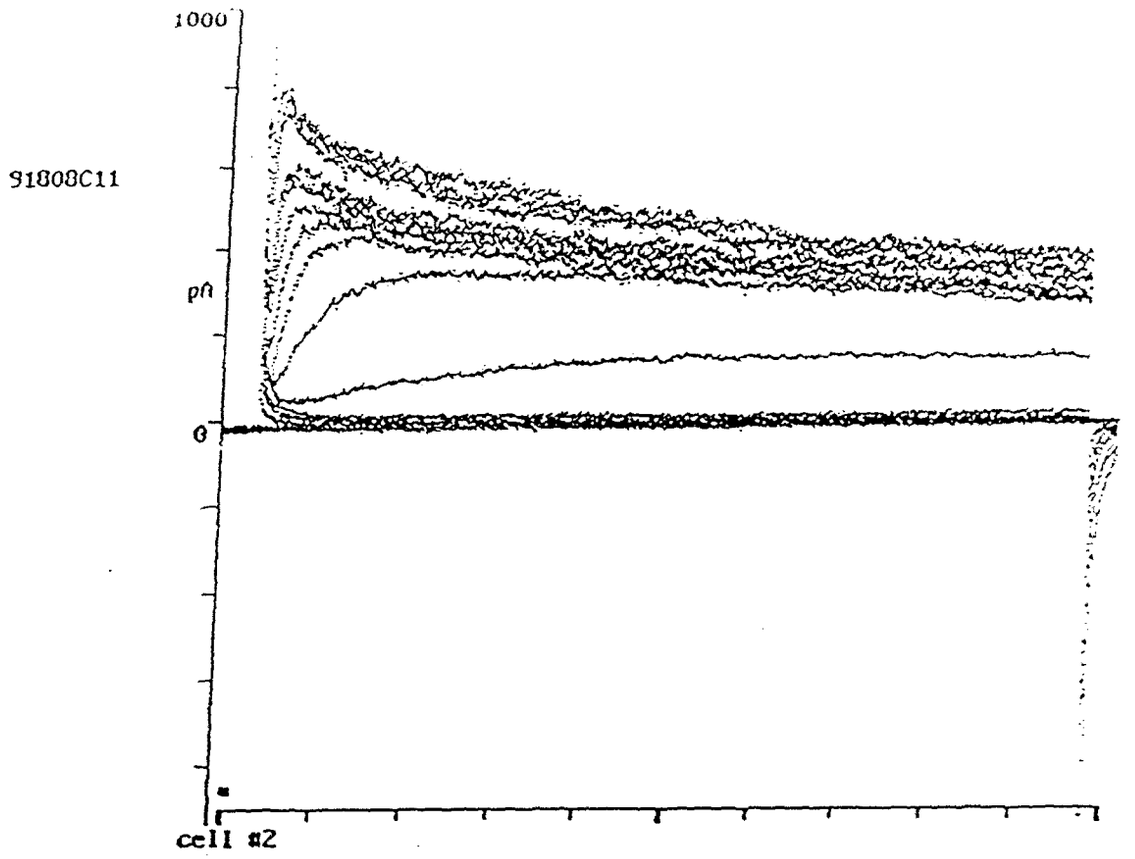


Fig. 1





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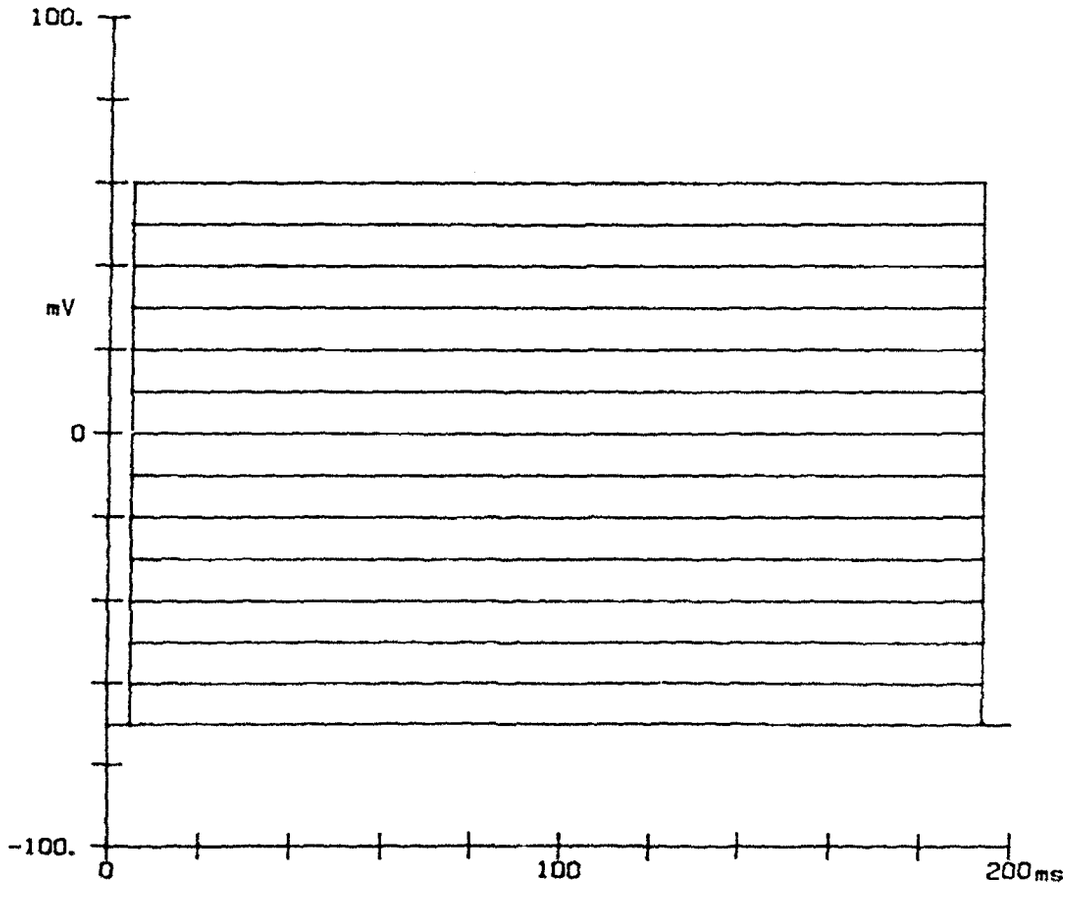

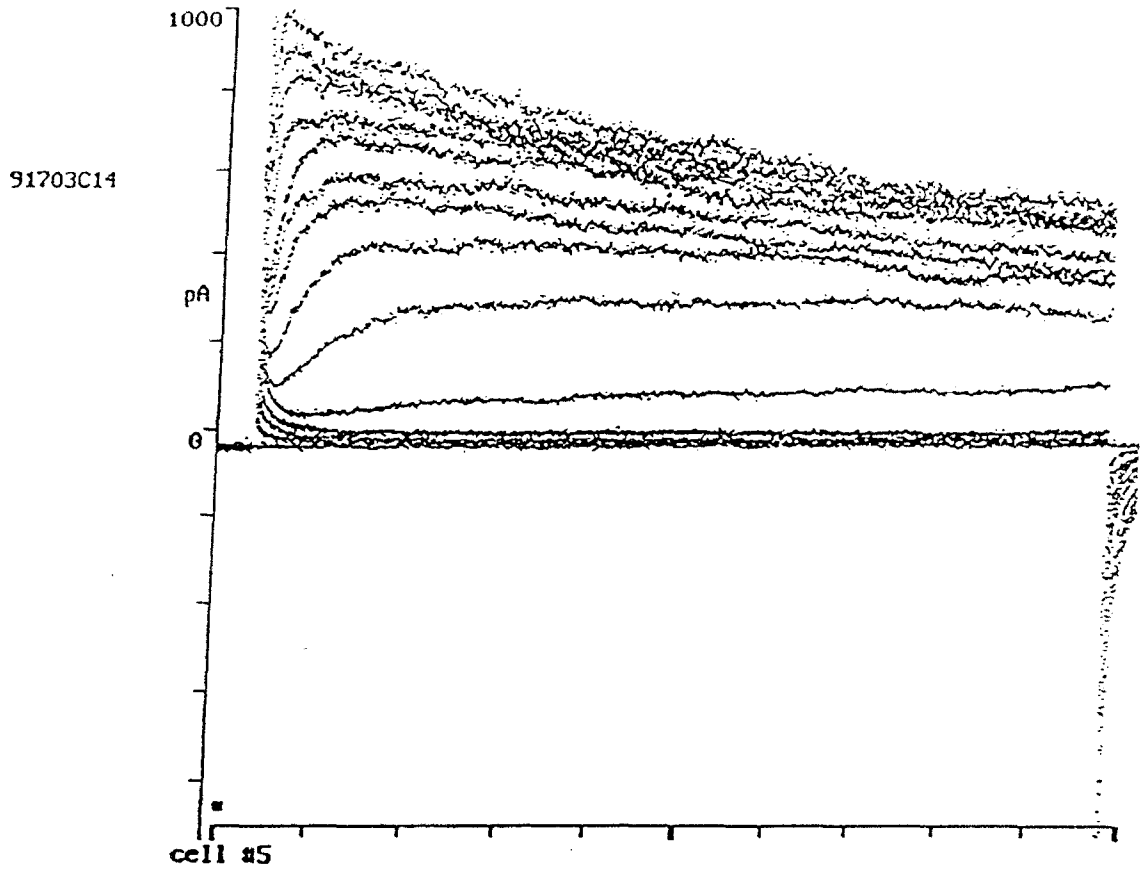

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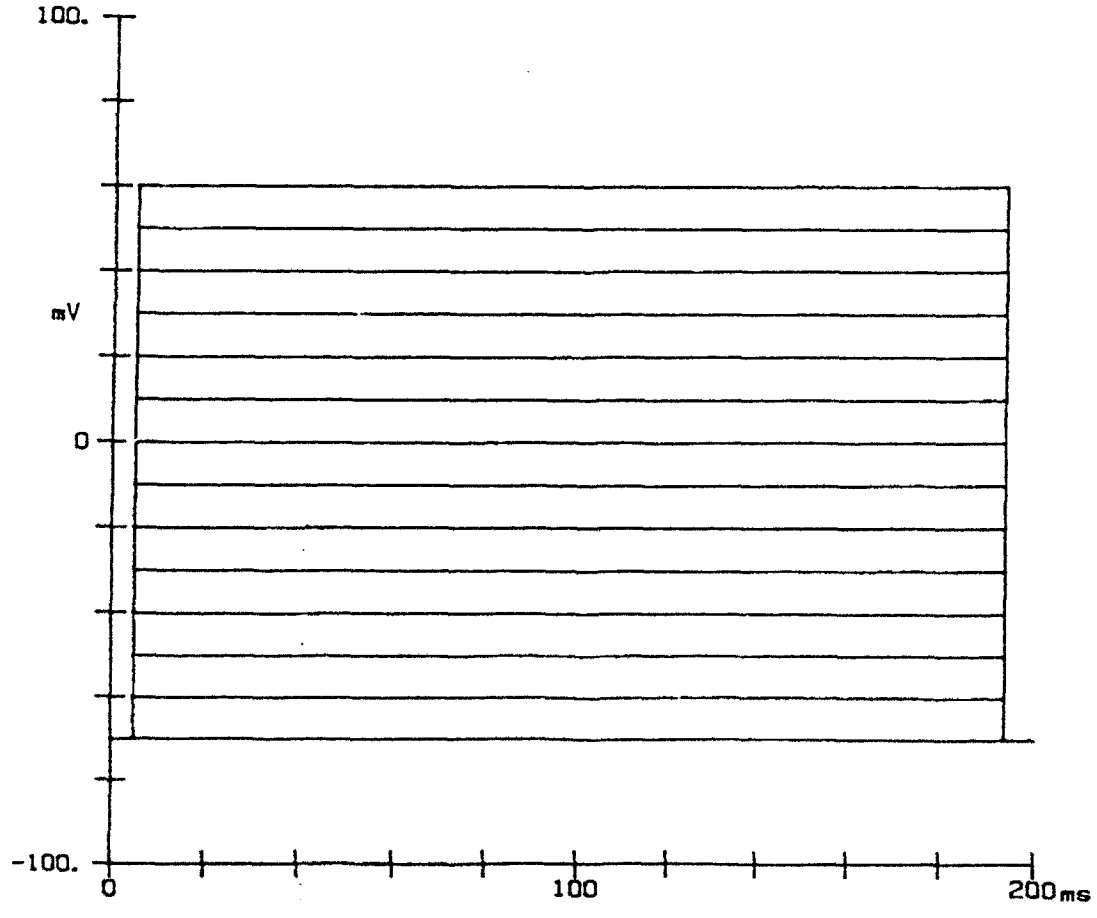

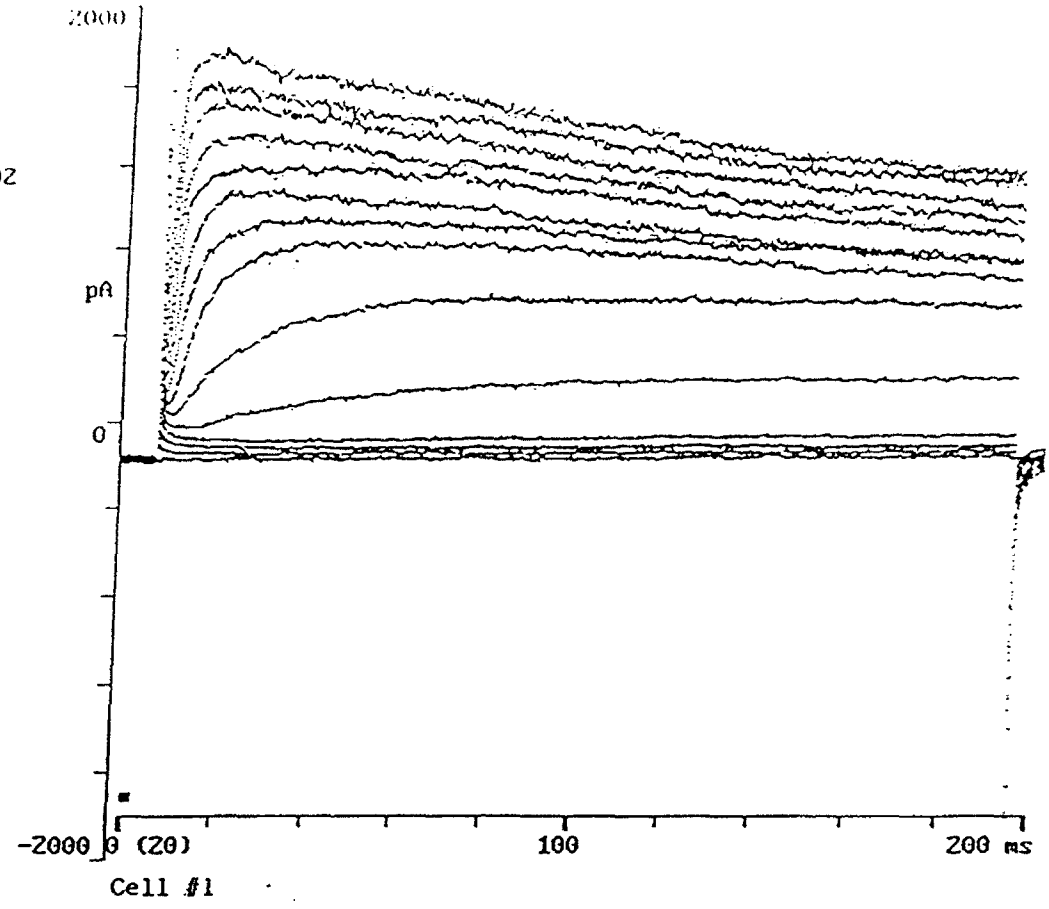
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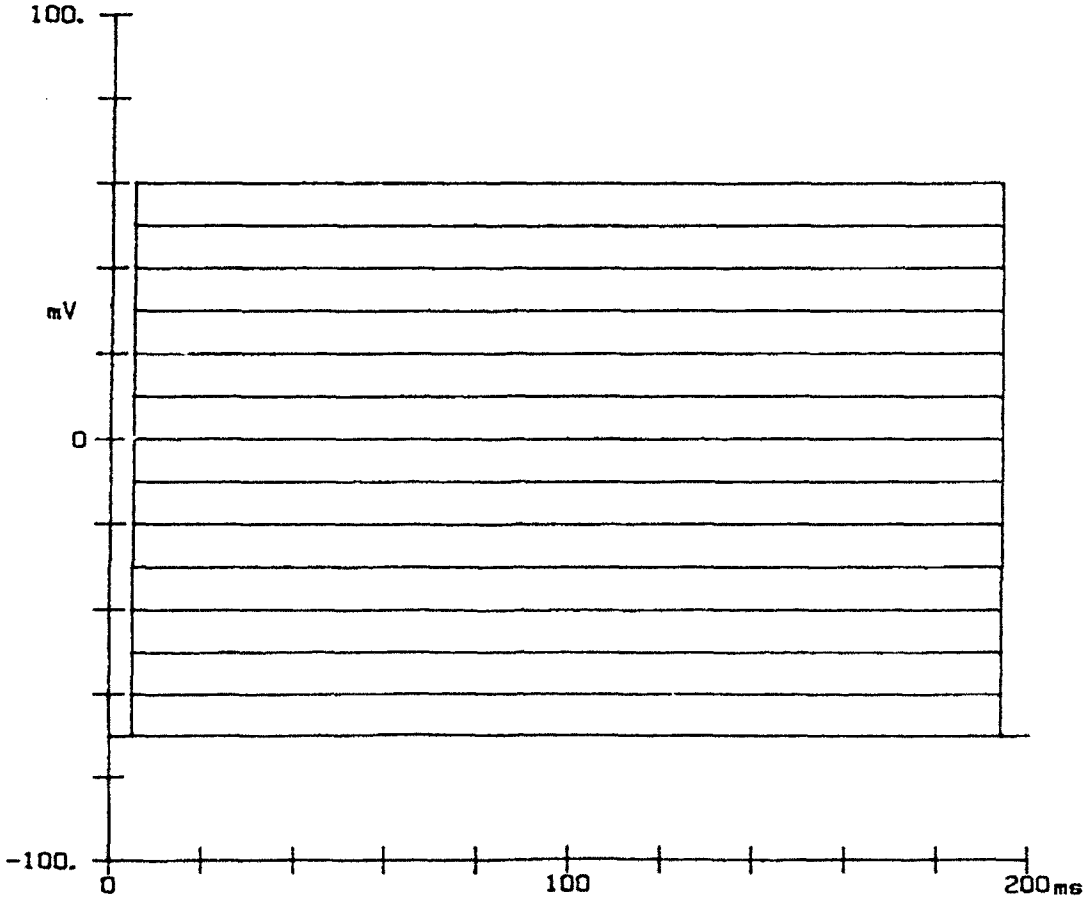
Fig. 4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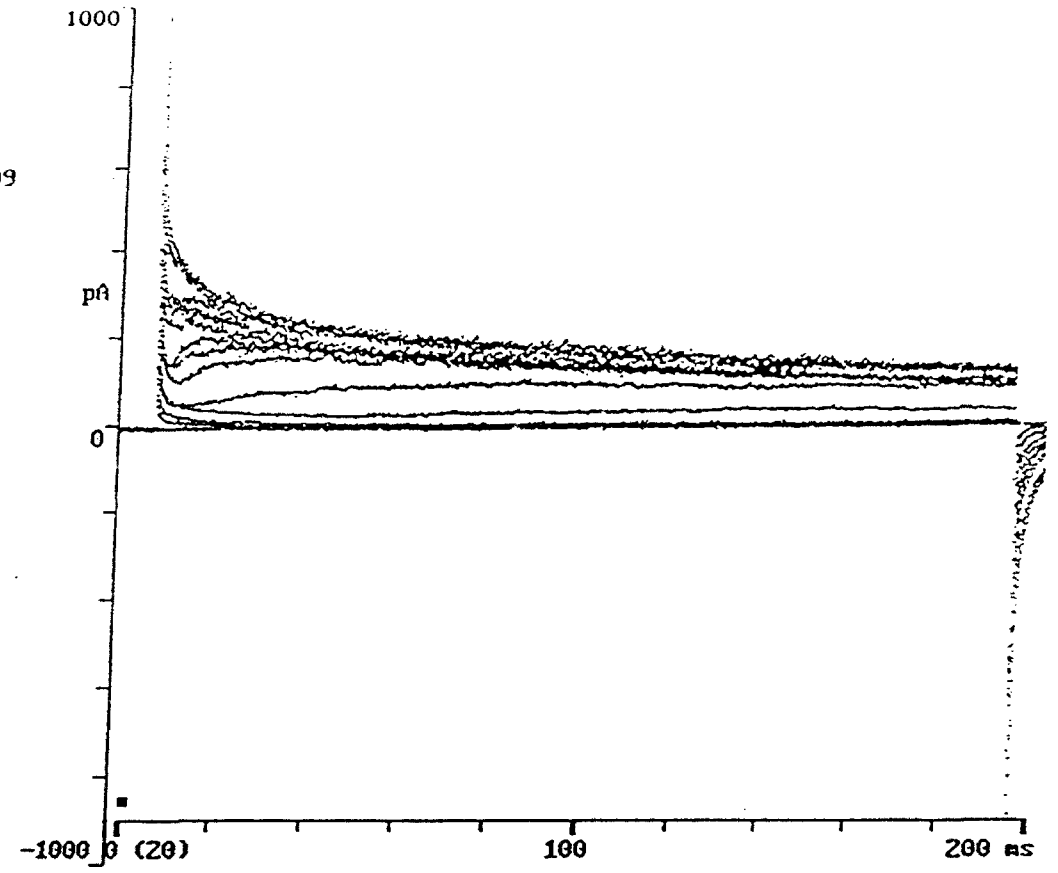
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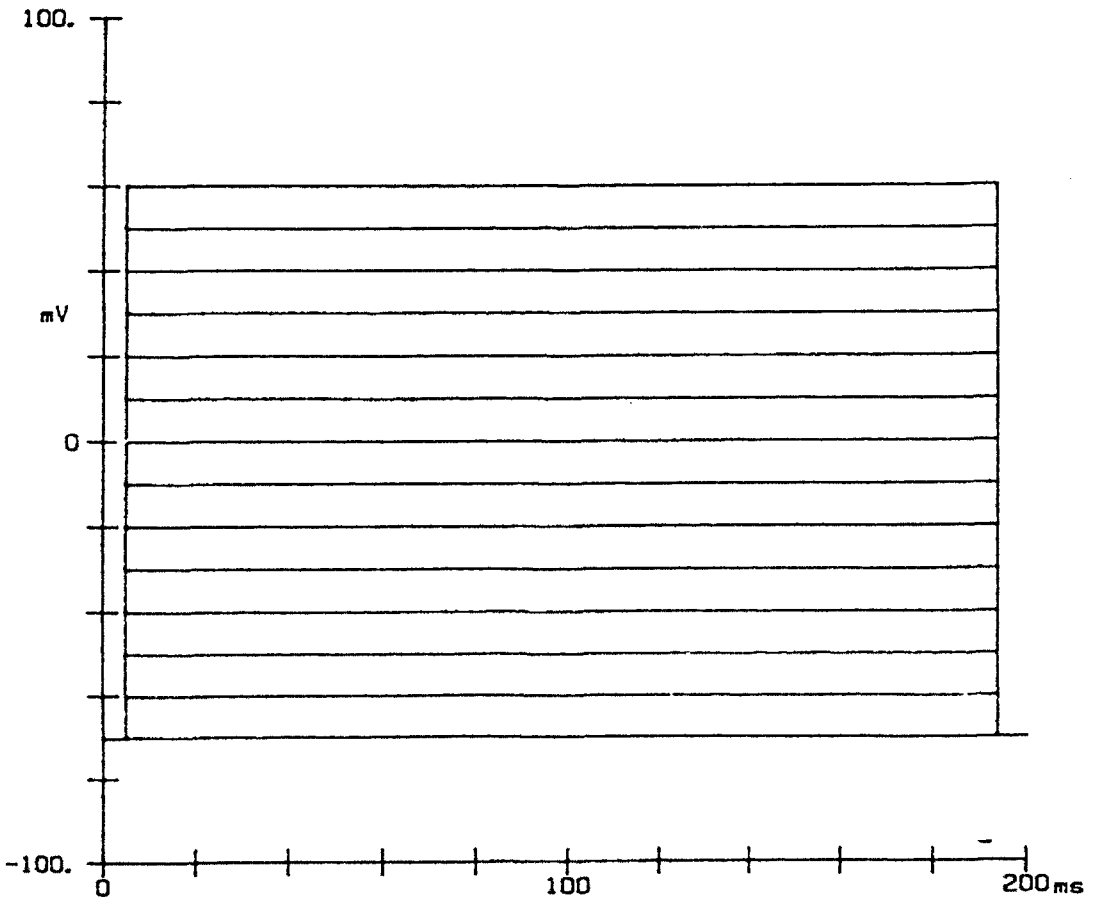
100
200 ms
Cell #2

Fig. 5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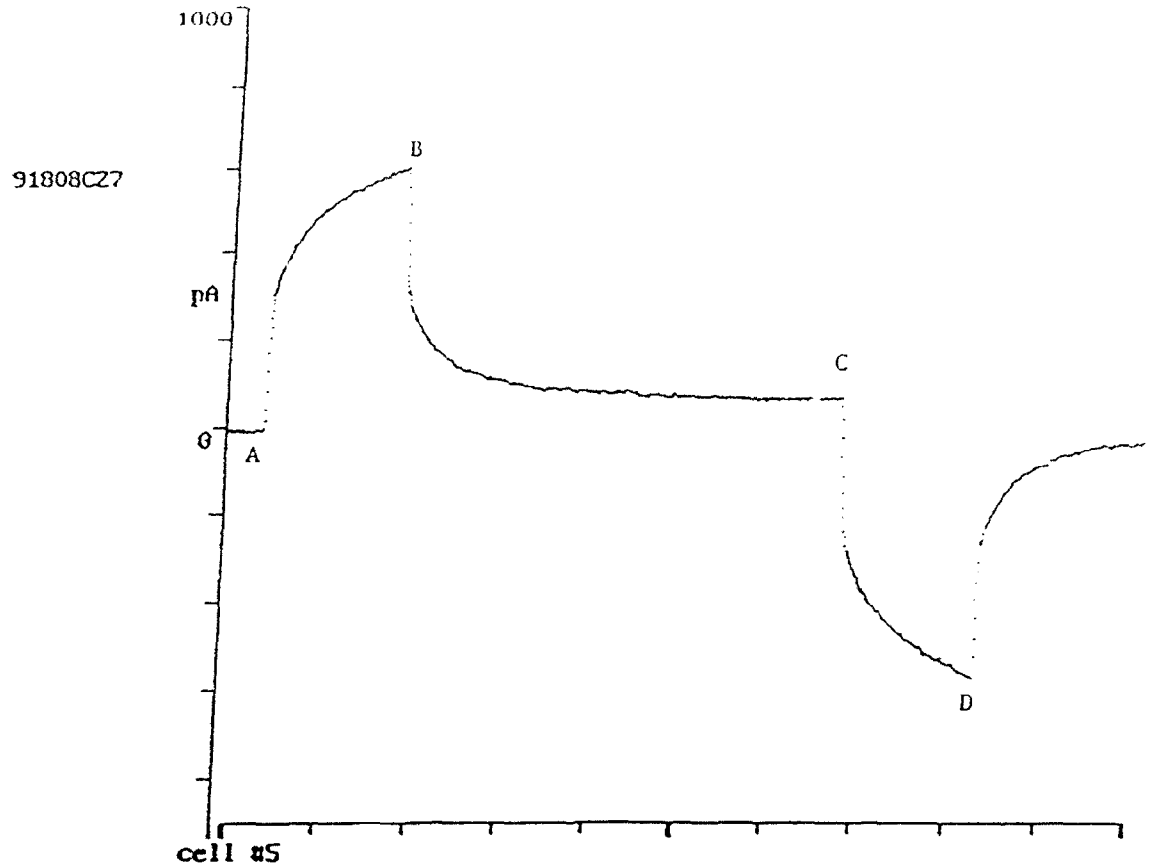
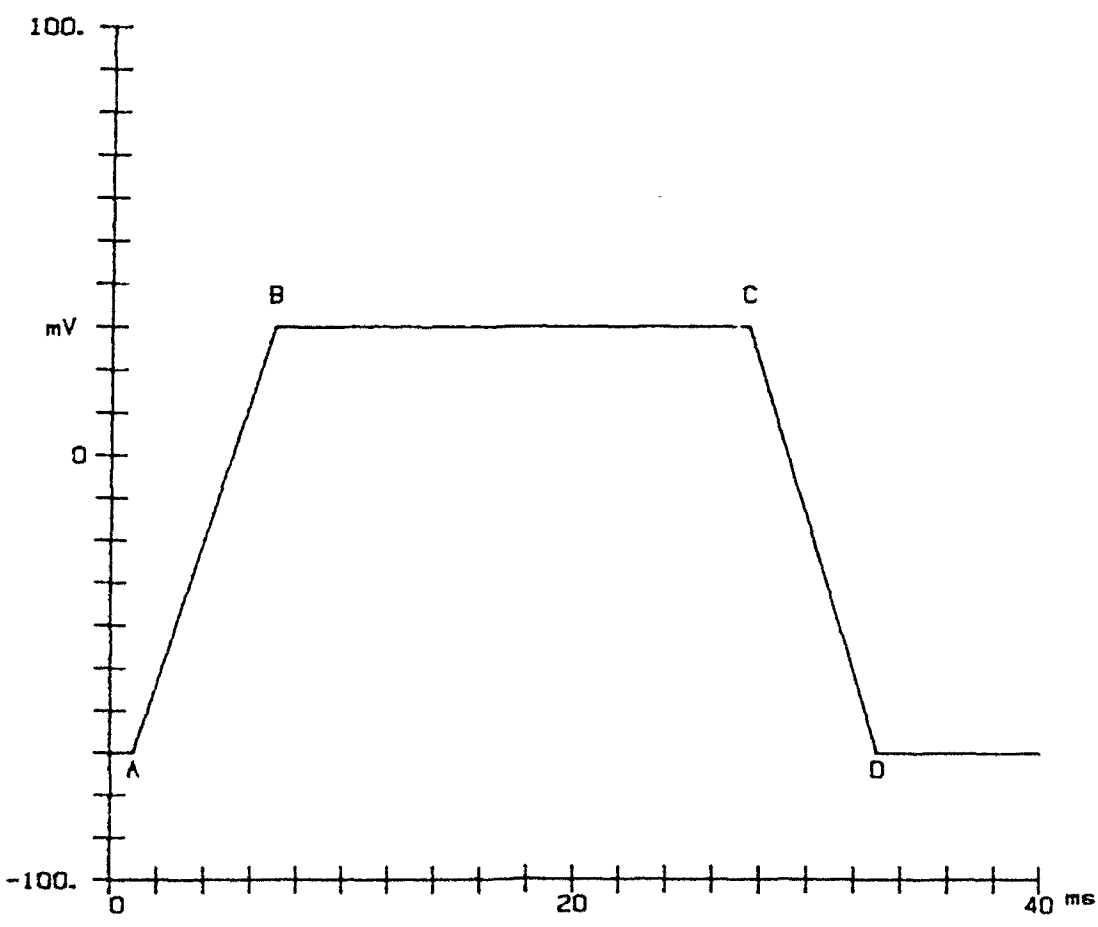


Fig. 6



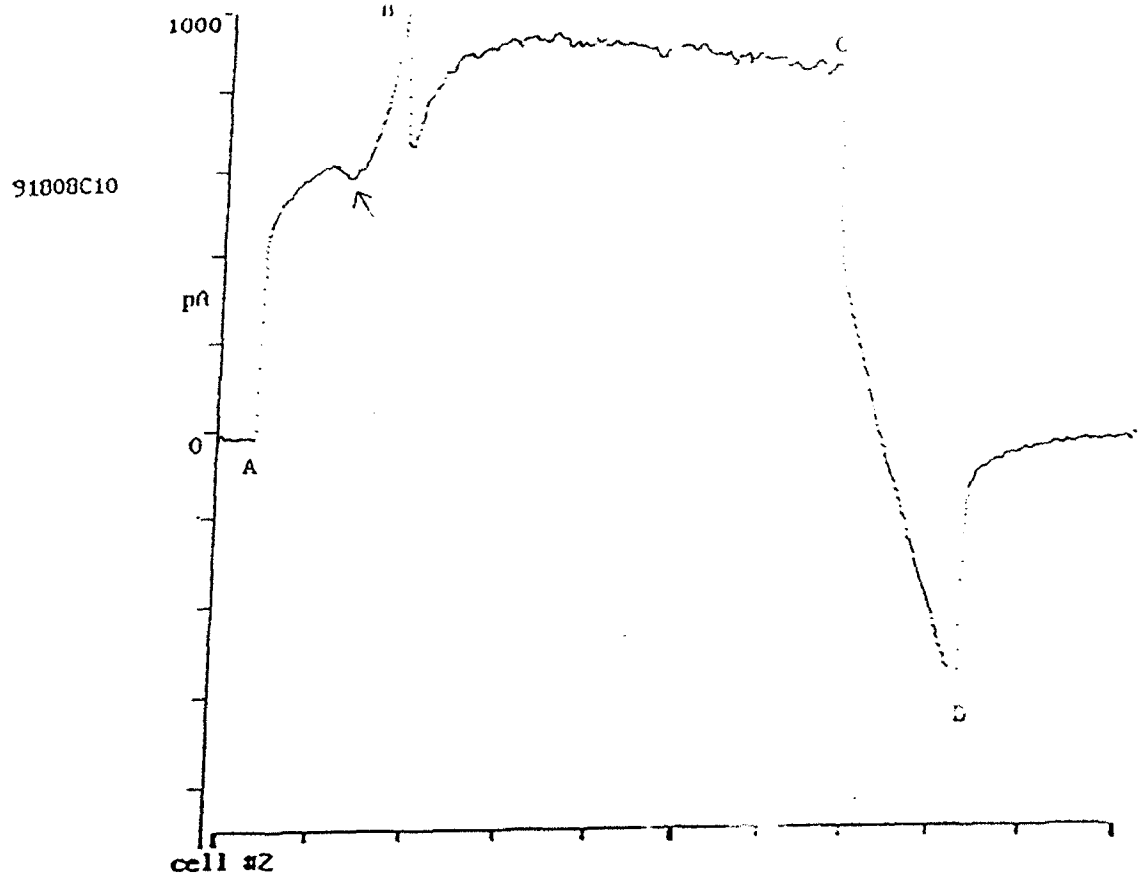
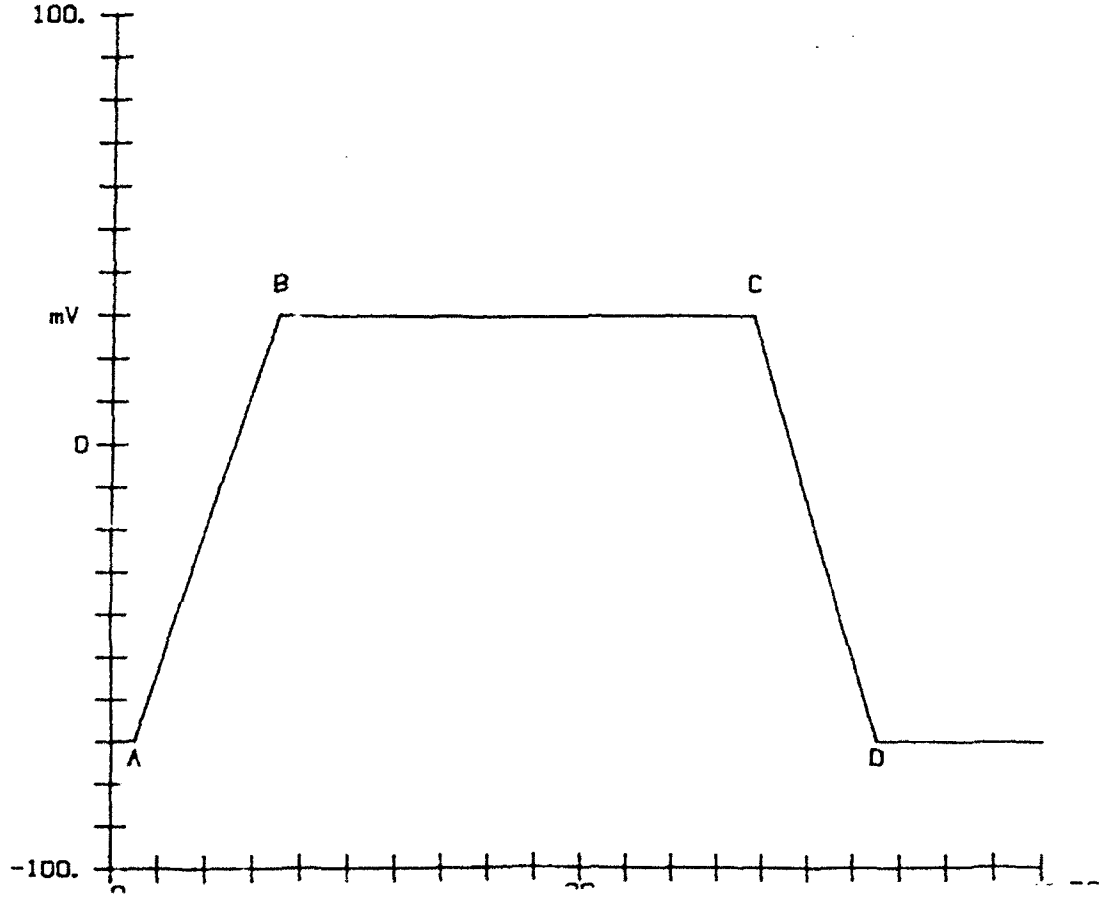


Fig. 7



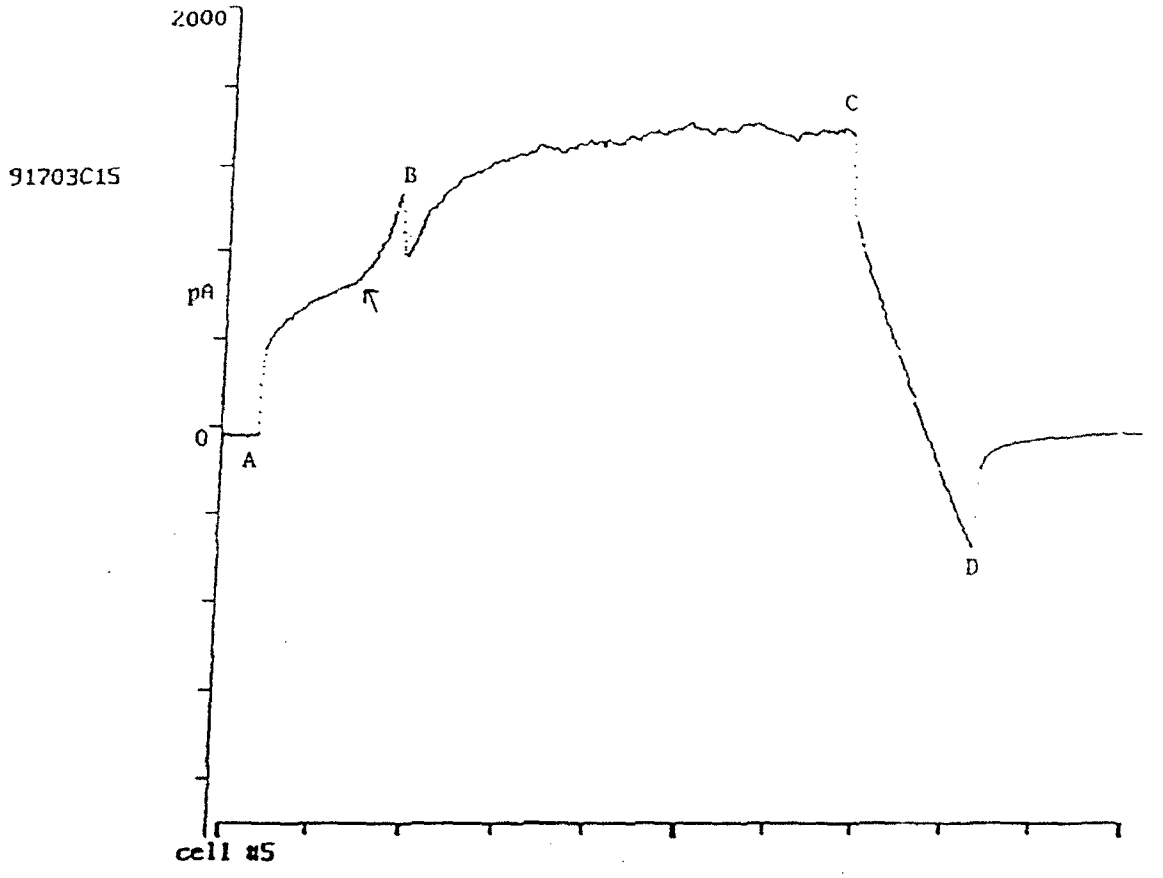


Fig. 8

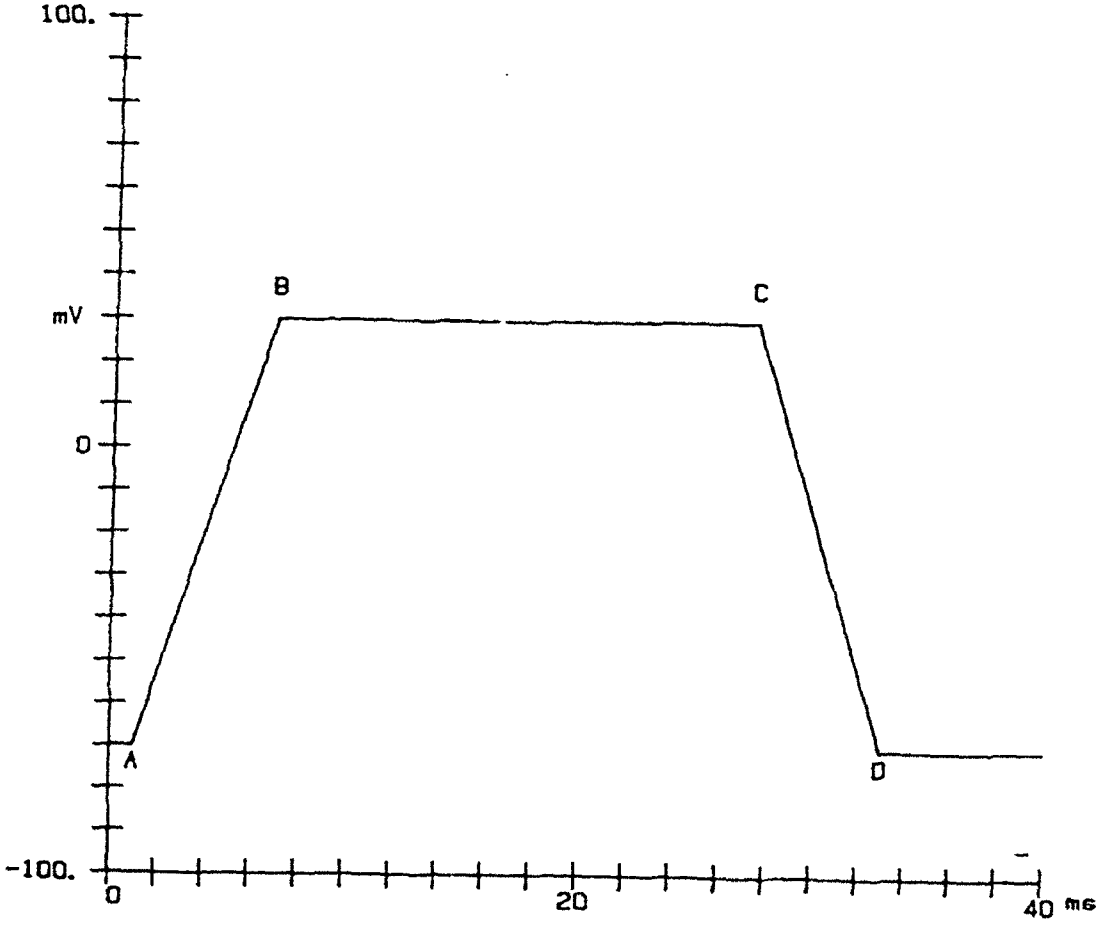


Fig. 9

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